Investigating the Mechanism Between Claudin-5 Reduction and the Development of Cardiomyopathy

Senior Thesis

Jordan Michelle Chambers
4-3-2014

School of Health and Rehabilitation Science
Biomedical Science Major
Senior Honor Thesis

Contents

Acknowledgments

I would like to recognize multiple people for their support, encouragement, and guidance throughout my undergraduate career. First, I would like to acknowledge and thank Dr. Jill Rafael-Fortney for her guidance as a mentor throughout my time in her lab. I would not have grown as much as I have as a scientist without your patience, guidance, and support. I truly appreciate all of the time you have dedicated to me during these past few years. I would like to thank Dr. Paul Janssen and his lab, particularly Mohammad Elnakish, for their time, expertise, and collaboration on this project. I would also like to recognize the other members of the Rafael-Fortney Lab and the claudin-5 team, who have assisted and supported me. Thank you to Dr. Dawn Delfin, Sarah Swager, Jennifer DeSalvo, Eric Schultz, Kevin Schill, Jason Murray, Neha Rastoogi, Jessica Chadwick, Slesha Thoma, Jenny Lee, and Lauren Chen.

I would also like to thank my parents, Jim and Julie, and my brother, Jared, for their continual support and faith in me. They have guided me every step of the way and without their encouragement I would have been lost.

Finally, I would like to thank my friends and sorority sisters for always being there to support me in any way I needed. Your patience and support means the world to me.

Vita

June 2010……..…Lake Zurich High School

March 2012-present…….....…Undergraduate Research Assistant

Rafael-Fortney Lab, Molecular and Cellular Biochemistry, the Wexner Medical Center at The Ohio State University

May-July 2013…….….Undergraduate Research Office Fellow, The Ohio State University

May, 2014……..…….....……B.S Biomedical Science, The Ohio State University

Fields of Study

Major Field: Biomedical Science Honors Research Distinction

Minor Field: Integrative Approaches to Health and Wellness

Abstract

Heart disease is the number one cause of death in the United States. Despite the prevalence of heart failure in the population, little is known about its molecular mechanism of heart failure. The tight junction protein claudin-5 has recently become of interest to my lab and others in heart failure. Claudin-5 has been shown to localize to the lateral membrane of cardiomyocytes and claudin-5 is reduced in the hearts of the dko (dystrophin/utrophin knockout) mouse model of Duchenne Muscular Dystrophy, which exhibits the clinical signs of cardiomyopathy. Claudin-5 has also been shown to be reduced in at least 60% of human end-stage heart failure. When claudin-5 levels were maintained in the hearts of dko mice, much of the cardiomyopathy was prevented. These findings indicate that claudin-5 is a potential therapeutic target for the treatment of cardiomyopathy and heart failure. To determine the mechanism between claudin-5 reduction and the development of cardiomyopathy, my lab developed an inducible cardiomyocyte-specific claudin-5 knockdown mouse model, where claudin-5's lone exon was excised using a Cre recombinase, which was activated by tamoxifen injection. At 14 weeks of age experimental mice and cre transgenic control mice exhibited reduced ejection fraction, indicating that a cre-dependent toxicity was occurring. To alleviate this issue, the mice were switched to tamoxifen citrate chow, which has been shown to cause less toxicity. At 14 weeks of age, experimental mice had an average ejection fraction of 57% while cre transgenic control mice had an average ejection fraction of 59%, which supports that the tamoxifen chow prevented the development of a Cre-dependent toxicity. The experimental mice have developed abnormal patterns in the basal lamina and they have developed areas of fibrosis in the extracellular matrix. Staining of an endothelial cell marker, CD31, shows that

experimental mice have developed abnormal wavy patterns of the endothelial cells. These findings indicate that claudin-5 is involved in the mechanical attachment of the cardiomyocytes to the extracellular matrix or to endothelial cells, since claudin-5 is also present in endothelial cells. This mouse model indicates that claudin-5 has a significant connection to the development of cardiomyopathy, which can be further explored using this model.

List of Tables and Figures:

Introduction

Dilated Cardiomyopathy

Dilated Cardiomyopathy is one of the most common causes of heart failure. The estimated prevalence of dilated cardiomyopathy is 1:2500[1]. There is a survival rate of less than 50% for patients who have exhibited dilated cardiomyopathy for greater than ten years. While the disease mostly affects those in their third and fourth decades, it can affect those of all ages[1]. This is a chronic illness that often leads to severe heart failure. Dilated cardiomyopathy results from an increase in the size of the left ventricle, which weakens the myocardium and prevents the heart from efficiently pumping, resulting in reduced ejection fractions[16].

There is no cure for dilated cardiomyopathy and the current standard of care is designed to prevent the progression of the disease. There are a variety of medications used to slow the disease. ACE (angiotensin-converting-enzyme) inhibitors and Angiotensin-II receptor antagonists are both designed to lower blood pressure, control the symptoms of heart failure, and slow the progression of the disease[16]. Beta-Blockers slow heart rate, which reduces the work load of the heart[16]. Calcium channel blockers increase the length of the resting phase of the heart by decreasing the amount of calcium entering the cardiomyocytes, also reducing the workload of the heart[16]. For more severe cases, pacemakers and ICDs (implantable cardioverter defibrillator) are implanted to control the heart rate and prevent arrhythmias[16]. For the most severe cases, heart transplants may be required.

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy is one of the more severe forms of the muscular dystrophies. The average lifespan of those affected is around 25 years old. The disease affects boys, since it is X-linked recessive. Duchenne muscular dystrophy is caused by the loss of the dystrophin protein[10]. This destabilizes the muscle membrane and results in atrophy of muscles. Cardiomyopathy and respiratory failure are the most common causes of death[13].

Duchenne muscular dystrophy is modeled in mice by two knockouts: *mdx* and dko. The mouse model has a mutation on the X chromosome, which results in a non-functional dystrophin protein. This model is genetically similar to the human disease. They develop weakness in muscles, but they do not develop many functional or histological signs of cardiomyopathy before six months of age[20]. The dko (or double-knockout) mouse has dystrophin and utrophin missing[23]. This mouse has a reduced lifespan and develops functional and histological signs cardiomyopathy by eight weeks of age[7]. They have decreased ejection fraction, increased left ventricle dilation, thinning of the ventricle and septum, and fibrosis occurs in their hearts[2,7]. My lab has studied the dko mouse model to determine gene expression changes that may account for the more severe pathology in dko compared to *mdx* hearts. They found that claudin-5, a transmembrane protein typically found in vasculature and endothelial cells, was reduced in dko mouse hearts[21].

Claudin-5

Claudin-5 is a transmembrane protein that is a component of tight junctions in endothelial tissue[17]. Knockouts of claudin-5 in mice result in neonatal death presumably

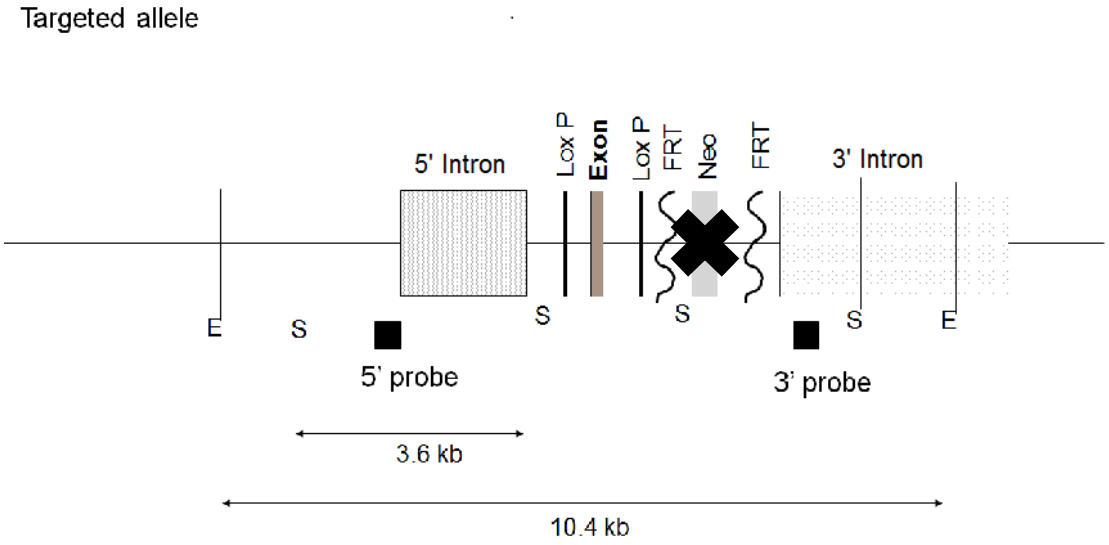
due to a size-selective loosening of the blood brain barrier[19]. However, the heart was never analyzed in this study. My lab discovered that claudin-5 also localized to the lateral membrane of cardiomyocytes[21]. They also found that claudin-5 is reduced in dko mouse hearts and this reduction results in an abnormal wavy pattern of the lateral membranes of cardiomyocytes[21]. This is particularly interesting, because out of the 24 different claudins, claudin-5 was the first to be found in the heart. Only claudin-12 is also present in the heart, but only at very low levels[8]. My lab also found that claudin-5 is reduced in at least 60% of human end-stage heart failure[15]. Claudin-5 reduction was independent of connexin-43, which had previously been shown to be reduced in failing heart samples[5]. Claudin-5 was also reduced independent of dystrophin. This finding indicated that claudin-5 was involved in a novel pathway to end-stage heart failure. My lab showed that claudin-5 could be used to rescue cardiomyopathy in dko mice[4]. Claudin-5 levels in the dko mice were sustained using an adeno-associated virus. They found that this expression prevented the development of the physiological and histological signs and symptoms of heart damage. Moving forward, my lab developed an inducible claudin-5 knockdown mouse model to determine if the reduction of claudin-5 alone could impair heart function.

MerCreMer-Lox System

The claudin-5 knockdown mouse was developed using the MerCreMer-Lox system. An inducible, cardiomyocyte specific knockdown model was developed, which was designed to excise claudin-5's lone exon from chromosome 16. Two loxP sites were inserted so that they flanked the claudin-5 exon. The Cre recombinase protein binds to these sites and excises the exon through deletion, inversion, or translocation. This "floxed"

construct was transfected into 129SV mouse embryonic stem cells. A neomycin antibiotic resistance cassette was inserted into the floxed construct to select cells containing the construct. Neomycin resistant cells were then screened for the correct homologous recombination event of the floxed construct replacing the wild-type claudin-5 allele. These cells were implanted into the blastocysts from pregnant mice. The neomycin cassette was flanked by two FRT sites, which allowed for the removal of the neomycin cassette with the use of flp recombinase. The mice expressing the claudin-5 construct were bred with mice expressing the flp recombinase to remove the neomycin construct. Mice expressing the construct, without the neomycin cassette, were bred with mice expressing Cre recombinase. The Cre recombinase and Flp recombinase expressing mice were purchased from the Jackson laboratory[18]. This Cre recombinase is driven by the myosin heavy chain 6 promoter, which drives expression of Cre recombinase to affect cardiomyocytes. The Cre recombinase was flanked by two mutated estrogen response elements (MerCreMer), which prevents Cre recombinase from entering the nucleus of the cell. These mutated estrogen response elements only respond to non-endogenous estrogen analogs, such as tamoxifen. The Cre recombinase is only able to enter the nucleus when the estrogen agonist tamoxifen binds to the mutated estrogen response elements. Once tamoxifen is either injected or ingested, the Cre recombinase enters the nucleus in cardiomyocytes and begins to excise the claudin-5 exon from chromosome 16.

Figure 1: Diagram of the target claudn-5 loxP allele to create claudin-5 knockdown mice. A mutated Cre recombinase binds to LoxP sites flanking the exon. This Cre recombinase is activated by estrogen agonists, such as tamoxifen, due to the presence of Mer sites around it. Once activated it moves to the nucleus and excises claudin-5 from chromosome 16. A neomycin cassette was present in the construct so that appropriate cells could be selected for, using antibiotic resistance. The neomycin cassette was removed using the Flp recombinase, which binds to the FRT sites flanking it.



Theoretically, homozygous floxed/floxed mice should be the experimental mice. However, there has never been a living, floxed/floxed experimental mouse with the neo cassette removed. There were floxed/floxed mice with the neo cassette present. However, the neo cassette interfered with the Cre excision, as is typical in many targeted alleles, preventing any reduction of claudin-5 from occurring. Since claudin-5 is a very small gene, the deletion of neo from the 3' intron could potentially affect CDC45 whose 1st exon is 2.1 kb downstream from Cldn5, which would result in the neonatal death of the floxed/floxed mice. CDC45 is required for postimplantation mouse development[23]. Heterozygous CDC45 knockouts live to a normal age and show no defects though. Heterozygous Cldn5 flox cre+ do show a phenotype and are the experimental mice in this project. Heterozygous

Cldn5 flox cre+ mice are likely a better model for human heart failure, since claudin-5 is only reduced in human heart failure, rather than being completely gone.

Methods

Breeding

All animal experiments were conducted under an approved IACUC protocol. Heterozygous Cre+ (Cldn5flox+/-; Cre+/-) were bred with wild type Cre- (Cldn5flox-/-; Cre-/-) mice. This produced litters with half heterozygous mice and half wild type mice. It also produced litters with 50/50 cre+ and cre- mice. This provided a variety of controls for the experimental heterozygous cre+ mice. However, this proportions became skewed to heterozygous mice, for some unknown reason. To reestablish the number of wild type mice, I backcrossed the line to C57 Black-6 (C57 BL/6) mice. This backcross was accomplished by mating heterozygous cre+ mice with C57 Bl/6 mice. This provided the correct proportions of genotypes (50/50 heterozygous/wild type and 50/50 cre+/cre-). Also, to easily track the litters of mice, each litter was named alphabetically. Thus the first litter born, under this naming system, was named Ariel and the next litter was named Baloo. All litters A-J were injected with tamoxifen, while all litters from K (Kanga) on were fed tamoxifen through their chow.

Polymerase Chain Reaction

Polymerase chain reactions were used to genotype the mice to determine which were experimental mice (heterozygous Cldn5 flox+/-; cre+/-, from here referred to as het cre+), control mice (heterozygous Cldn5 flox+/-; cre-/- (het cre-), wild type Cldn5 flox-/-; cre+/- (WT cre+), and wild type Cldn5 flox-/-; cre-/- (WT cre-)), and appropriate breeding mice.

At three weeks of age approximately a centimeter of tail was snipped from the mice and digested in 600 µl of TNES buffer (0.5 M Tris-HCL pH 7.5, 5 M NaCL, 0.5M EDTA, 10% Sodium dodecyl sulfate) and 17.5 µl of 20 mg/ml proteinase K. These were incubated overnight at 55°C in a rotating oven. If the tails did not digest after an overnight incubation, another 50 µl of proteinase K was added. Once the tails had fully digested, a salt-out extraction was used to isolate the DNA. 167 µl of 5 M NaCl was added to each tube. The tubes were shaken for 15 seconds. They were then centrifuged for 10 minutes, at 4°C, at 14K rpm. The supernatant was poured into a new tube and 800 µl of 100% ice-cold ethanol was added to each tube and the samples were gently mixed. The DNA was then spooled onto a closed-end Pasteur pipette. The pipette was rinsed in 70% ethanol. The DNA was then resuspended in 300 µl 1X TE buffer (10 mM Tris-HCl pH 8.0, and 1mM EDTA) and incubated for 10 minutes at 65 °C to aid dissolution. They were stored in this buffer at 4 °C. DNA was then genotyped using PCR reactions with the CreER primers (For: CCGGTCGATGCAACGAGTGAT, Rev: ACCAGAGTCATCCTTAGCGCC 59°C annealing temperature), 5'LoxP primers (For: ACCAGAGTCATCCTTAGCGCC, Rev: CGAAGTTATTAGGTCCCTCGACC 62°C annealing temperature), and wild type primers (For: GGAGAAGAACCTACTGAACCAAAGG, Rev: TCACCCAAGTTGCCATTCCC 59°C annealing temp). These primers detected the presence of the Cre transgene, the floxed construct, and the wild type product respectively. The PCR reaction contained 7.5 µl 2X Syzygy Taq master mix, 0.3 µl forward primer, 0.3 µl reverse primer, 5.9 µl distilled water, and 1µl of DNA. The products were separated by gel electrophoresis. ΦX HaeIII was used as the molecular weight marker. The presence of a PCR product at 798 bp indicated the presence of the Cre recombinase gene. The presence

of the 5'loxP product was determined by the presence of a ~600 bp product when the reaction was run with 5'loxP primers. This product indicated that at least one allele contained the 5'loxP construct. A wild type primer reaction was used to determine the presence of the wild type allele. The WT product was ~550bp. Heterozygous mice show two products, one for the WT product and one for the 3'loxP product. PCR reactions were visualized using 1% (5'LoxP and Cre PCRs) or 2% (wild type PCRs) agarose gels.

Tamoxifen Injection

At 4 weeks of age the mice were injected with tamoxifen (Sigma) in corn oil at 20 mg/ml. Each mouse was weighed before injection. The mice were administered 200 mg/kg per day of tamoxifen per weight. The tamoxfien was administered with a intra-peritoneal injection for 5 consecutive days.

Tamoxifen Chow

Due to toxicity issues, tamoxifen began to be administered by chow, rather than injection, as per discussion with the investigator who originally generated this Cre strain. The mice were still started at 4 weeks of age. There was 400 mg/g of tamoxifen citrate/kg Teklad Global 16% Protein Rodent Diet in chow. The mice were fed tamoxifen chow for two consecutive weeks, then switched to normal chow. The estimated dose per mouse was 40 mg/kg per day.

Echocardiograms

Each mouse underwent serial echocardiograms at 8 weeks of age, conducted by Mohammad Elnakish from Dr. Paul Janssen's lab. These were used to calculate left ventricle mass/body weight ratios and ejection fractions.

Dissections

Mice were dissected at 14 weeks of age. A blood sample was taken and spun down to collect serum. The heart was then removed and cut transversely into two sections. The apical third was frozen in liquid nitrogen for homogenization. The top two thirds of the heart were frozen in OCT for histological sectioning. The lungs, liver, and kidney were then removed and frozen in liquid nitrogen. A quadriceps was removed and cut transversely. One section was frozen in liquid nitrogen for homogenization and another was frozen in OCT for sectioning. The OCT samples were cut in to 8 µm sections using a cryostat, mounted on slides, and stored at -80 °C.

Tissue Analysis

Immunofluorescence

Heart sample slides were brought to room temperature and the sections were encircled with a hydrophobic pen to form a barrier. The slides were equilibrated in potassium phosphate buffered solution (KPBS-K_2HPO_4, KH_2PO_4, NaCl) for 5 minutes. They were blocked with KPBS + 1% gelatin for 15 minutes. They were rinsed with KPBSG (KPBS + 0.2% gelatin) for 5 minutes. They were incubated in 100 µl of the antibody of choice (see Table 1) for 2 hours, at room temperature, in a wet chamber. The antibody was diluted to the appropriate

dilution with KPBSG and 1% normal goat serum (NGS). The slides were rinsed for 5 minutes 3 times in KPBSG. 100 µl of the secondary antibody was then applied and the slides were incubated for 1 hour, at room temperature, in a wet chamber, in the dark. The secondary antibody was diluted to the appropriate dilution with KPBSG and 1% normal goat serum. The slides were then washed with KPBSG for 5 minutes 3 times. The slides were mounted with 35 µl vectashield and DAPI (at 2 µg/ml) and coverslipped. The slides were stored in a -20 °C frost free freezer. The sections were visualized using the Nikon Eclipse 800 microscope with an epifluorescence mercury lamp. The exposure time was determined based on the positive and negative control slides so that the staining could be seen, without being overly bright and kept consistent between all slides for a given experiment.

Table 1: Primary Antibodies used for immunofluorescent staining.

Antibody (Species)	**Company**	**Dilution**	**Other Specifications**
Claudin-5 (rabbit)	Abcam	1:200	None
Ephrin-B1 (goat)	R&D Systems	1:25	Fetal Bovine Serum was used instead of NGS
Tenascin (rabbit)	Millipore	1:100	None
Fibronectin (rabbit)	Abcam	1:40	None
Collagen (rabbit)	Abcam	1:100	None
CD-31 (rabbit)	Abcame	1:50	None

Collagen Quantitation

The amounts of collagen scarring was quantitated by measuring the immunoreactive area using Photoshop. 4x images were composited together in Photoshop and then fibrotic areas were selected and marked red. Using the histogram function in Photoshop, the percentage of red colored areas was calculated. This percentage represents the percentage of the heart section that contained collagen and values above control, which supports the presence of fibrosis.

Hematoxylin and Eosin Staining

Heart sample slides were also stained with hematoxylin and eosin to examine the structure and integrity of the heart tissue. The slides were fixed in 100% ethanol for 30 sec at room temperature. They were washed in tap water. The slides were then stained in hematoxylin for 30 sec to stain cell nuclei, then the slides were washed in tap water. They were stained in eosin for 20 sec to stain the cytoplasm of cells, then the slides were washed in tap water. They were then dehydrated in 100% ethanol, 90% ethanol, and 70% ethanol and then they were washed with histochoice clearing agent. The slides were then mounted with cytoseal and coverslipped. The slides were visualized using a light microscope. Composite pictures were taken at 4X magnification. The individual photos were composited together using Adobe PhotoShop.

Transmission Electron Microscopy (TEM)

An approximately 1 mm by 1 mm cube of the ventricle of the heart was taken from six mice (2 het cre +, 2 het cre-, 2 WT cre +). This cube was fixed in 3% glutaraldehyde and 4% PFA (25% glutaraldehyde, 4.5% PFA in 0.1M sodium phosphate pH 7.4) for 1 hour. The cube was then fixed in 3% glutaradehyde (25% glutaraldehyde in 0.1M sodium phosphate pH 7.4). The samples were then processed and image by the Campus Microscopy and Imaging Facility (CMIF) and viewed on a G2 Spirit TEM by an FEI technician.

Subcellular Fractionations

Whole hearts were weighed and resuspended in 7.5 x volume of homogenization buffer (20mM sodium pyrophosphate, 20mM sodium phosphate monobasic, 1 mM $MgCl_2$, 303

mM sucrose, 0.5 mM EDTA, HCl to raise pH to 7.1, leupepetin, pepstatinA, benzamidine, PMSF). The hearts were homogenized using a small homogenization probe. Between each sample the probe was rinsed twice with distilled water and detergent, just distilled water, and ethanol. Samples were centrifuged at 14,000 g at 5 °C for 15 minutes. The supernatant was pipetted off and transferred to another tube, where homogenization buffer was added until there was 2 ml of solution. Each tube was balanced to be the same weight and centrifuged for 37 minutes at 142,000 x g in a S55S Ti rotor in an ultracentrifuge cooled to 4 °C. The supernatant was pipetted off and then the pellets were resuspended in 2 ml of KCl-Wash buffer (0.6 M KCl, 0.3 M sucrose, 50 mM Tris-Cl pH 7.4, 0.1 mM PMSF, 0.75 mM benzamidine). Each tube was balanced to be the same weight. The samples were spun for 37 minutes, at 4 °C, at 142,000 x g using the S55S Ti rotor in an ultracentrifuge. The supernatant was poured off and the pellets were resuspended in 50 µl of resuspension buffer (10 mM sodium phosphate pH 7.8, 5 mM EDTA, 150 mM NaCl, leupeptin, pepstatinA, benzamidine, PMSF). These purified microsomes were stored at -80 °C.

Results

At 8 weeks of age the echocardiograms of mice injected with tamoxifen showed reduced ejection fraction for het cre + (52.97%) and wt cre + (46.93%) mice compared to het cre – (68.97%) mice (see figure 2). This indicated that the tamoxifen injection treatment resulted in some form of cre-dependent toxicity, which was detrimental to whole heart function. After these results, the mice were switched to tamoxifen chow, which has been shown to alleviate toxicity issues[11]. At 14 weeks of age het cre+ mice had an average ejection fraction of 57.3%, which is lower than any other genotype, including wt cre+

(59.1%) (see figure 3). Excision PCR was performed by another member of my lab, which showed that tamoxifen chow claudin-5 excision was as efficient as tamoxifen injection claudin-5 excision. Longitudinal echoes on the same mice are underway.

Figure 2: Ejection fractions of tamoxifen injected CKD mice at 8 weeks. Both wild type cre+ and heterozygous cre+ mice show reduced ejection fraction at 8 weeks of age. This indicates that the tamoxifen treatment produced a cre-dependent toxicity.

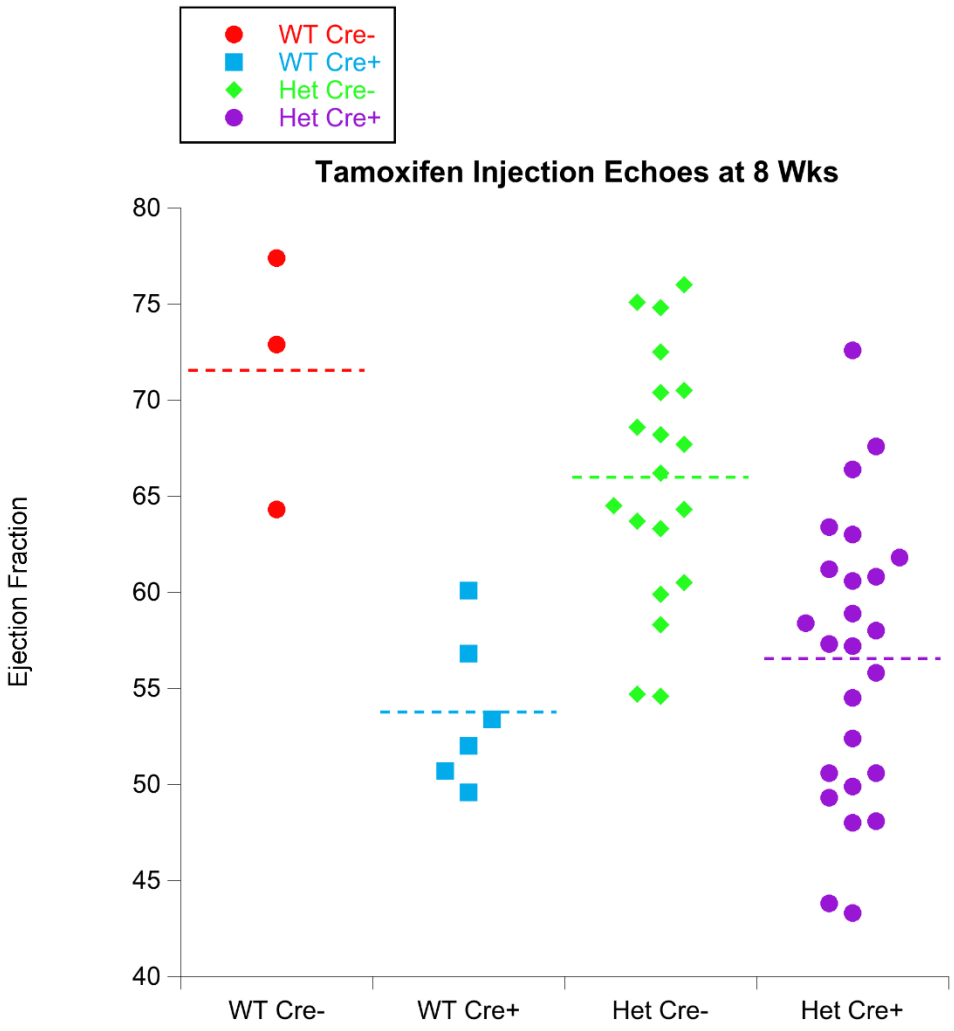
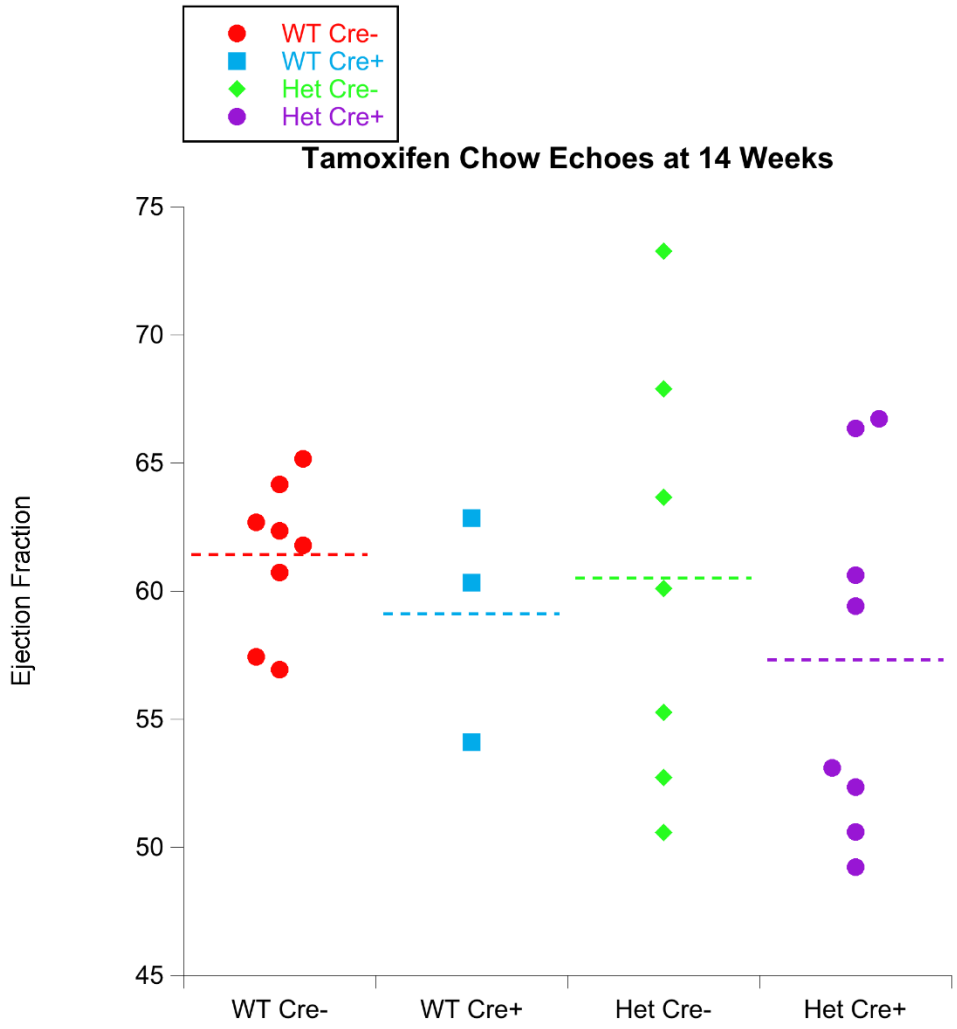


Figure 3: Ejection fractions of tamoxifen-chow mice at 14 weeks of age. The het cre+ mice have an average ejection fraction of 57%, which is lower than any other genotype.

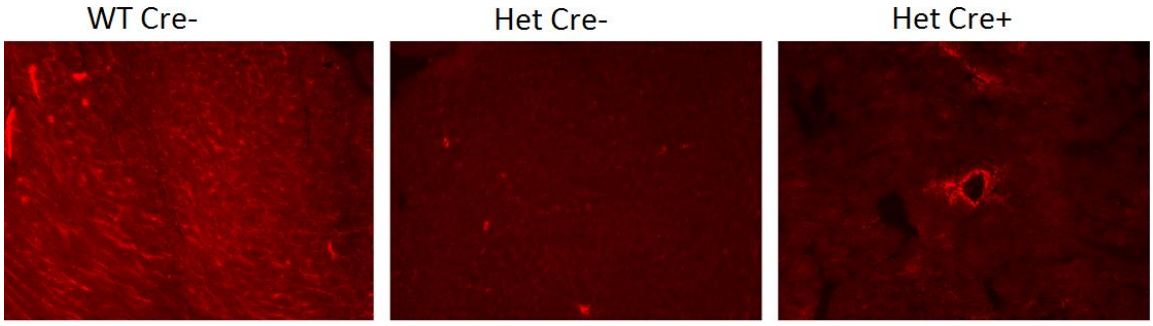


Hematoxylin and Eosin staining was used to create composites of whole mouse hearts. Comparison of these composites showed that the tamoxifen injected het cre+ mice had dilated hearts, with thinner walls (see figure 4). This finding has not been replicated yet by the tamoxifen chow fed mice and may have been caused by the tamoxifen toxicity.

Figure 4: Hematoxylin and eosin staining of claudin-5 knockdown by tamoxifen injection mouse hearts. The heterozygous cre+ heart is dilated, with thin walls and much irregularity in the papillary muscle structure. This has not been replicated with tamoxifen chow fed mice and may have been due to tamoxifen injection toxicity.

Claudin-5 immunofluorescence staining on 14 week old CKD mouse hearts showed a reduction of claudin-5 in the membranes of cardiomyocytes (see figure 5). Similar results are observed with both tamoxifen delivery methods. Claudin-5 was still normally expressed in vessels. The reduction in claudin-5 occurred in a mosaic pattern, with certain regions affected more severely than others. This is likely due to the fact that Cre excision of the claudin-5 allele is a stochastic event and may not occur in every cardiomyocyte.

Figure 5: Claudin-5 immunofluorescence staining on CKD mouse hearts. Claudin-5 has been reduced in the cardiomyocytes of the heart, but it is unaffected in the large vessels.



Ephrin-B1 is a transmembrane protein that has been found to bind with claudin-5 in cardiomyocytes[6]. Ephrin-B1 immunofluorescence staining showed that ephrin-B1 was also mosaically reduced in the lateral membrane of cardiomyocytes, in a pattern similar to claudin-5 (see figure 6). This indicates that ephrin-B1 stability requires claudin-5 and the two likely form a complex in the lateral membrane of cardiomyocytes.

Fibronectin is an extracellular matrix protein. In 14 week old CKD mouse hearts, fibronectin staining showed areas of irregular staining in the het cre+. There were many regions in the heart that had increased fibronectin staining in a scarring pattern (see figure 6). This indicates that the loss of claudin-5 allows the extracellular matrix to proliferate and scarring to occur. Other extracellular matrix proteins were also shown to have a similar pattern of staining in the het cre+ mice. Tenascin and collagen showed a very similar staining pattern to fibronectin. Composites of the whole heart were made for the collagen staining and the percentage of areas of scarring was quantitated (see figure 7). The het cre+ mice had a higher percentage of scarring in the collagen quantifications.

Figure 6: Immunofluorescence of CKD mice at 14 weeks of age. The top row is ephrin-B1 staining, which shows a reduction in the heterozygous cre+ mice at the cardiomyocytes. Ephrin-B1 was reduced in a mosaic fashion, similar to claudin-5. This is likely because the Cre excision is a stochastic event, which may not occur in every cardiomyocyte. The middle row is fibronectin staining, which illustrates the abnormal structure of the extracellular matrix. The heterozygous cre+ mice shows increased areas of scarring and irregular staining. The bottom row is tenascin, which is another extracellular matrix protein. This staining shows the same pattern as fibronectin, with increased areas of scarring in the heterozygous cre+ mice. Two het cre+ mice are shown to show the range in staining between our experimental mice.

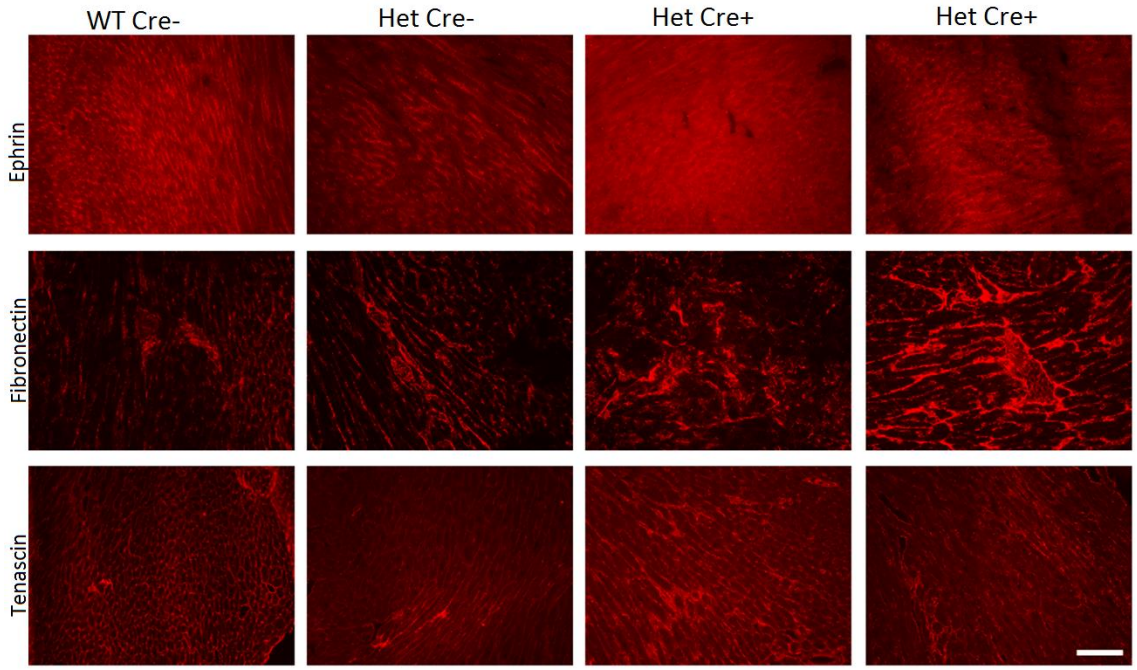
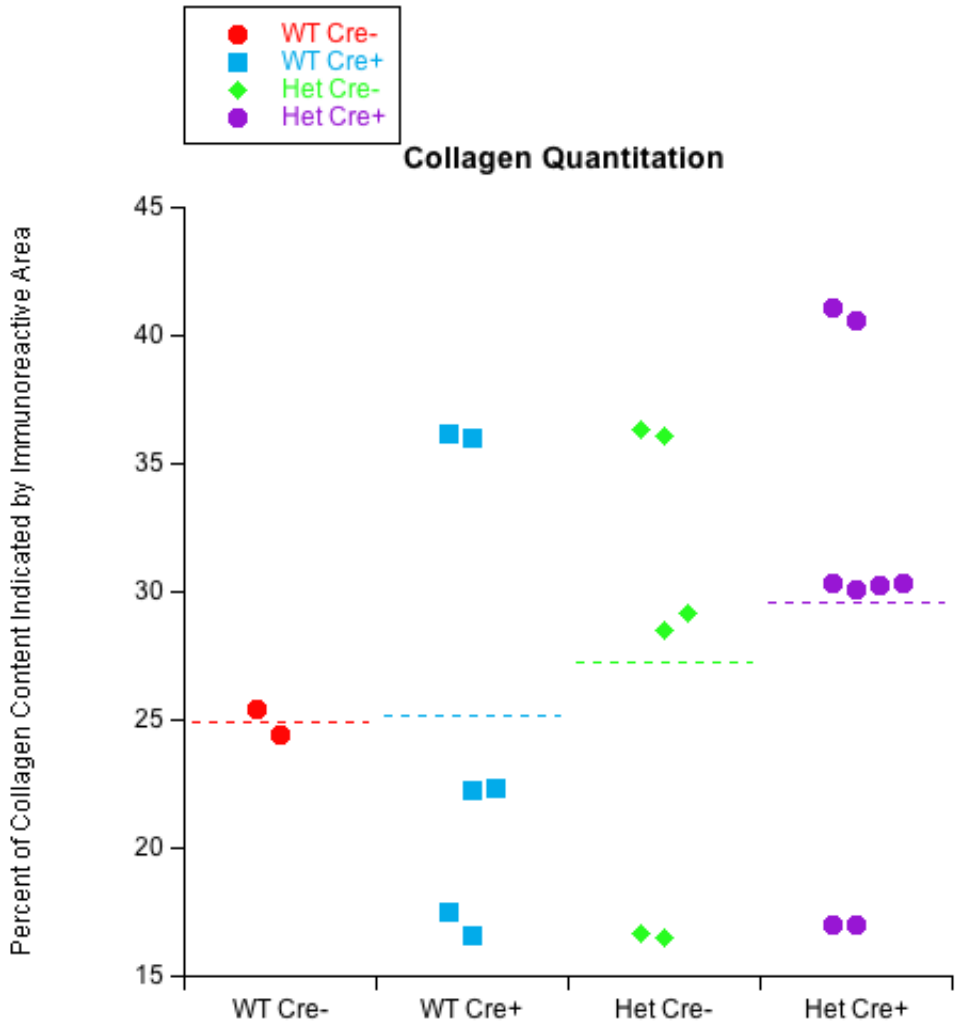


Figure 7: Collagen Quantification. The percentage of collagen scarring in the heart was calculated. Heterozygous cre+ mice had an increased percentage of scarring, compared to the other genotypes, including wild type cre+. This indicates that some fibrosis occurs in the knockdown mouse hearts, which is comparable to other mouse models of cardiomyopathy.



Transmission electron microscopy imaging on 14 week old CKD mouse hearts showed irregular patterning for the het cre + mice at the basement membrane. The membrane had lost electron density and normal basal lamina structure. Also, the

extracellular space between cells appeared increased. All other genotypes of mice appeared normal (see figure 8).

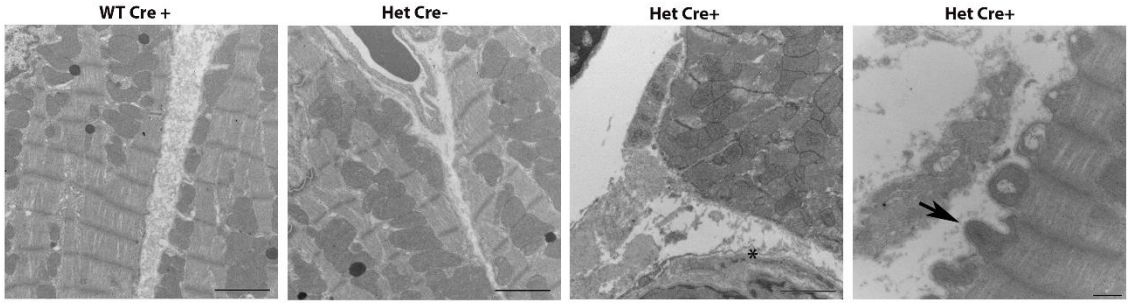
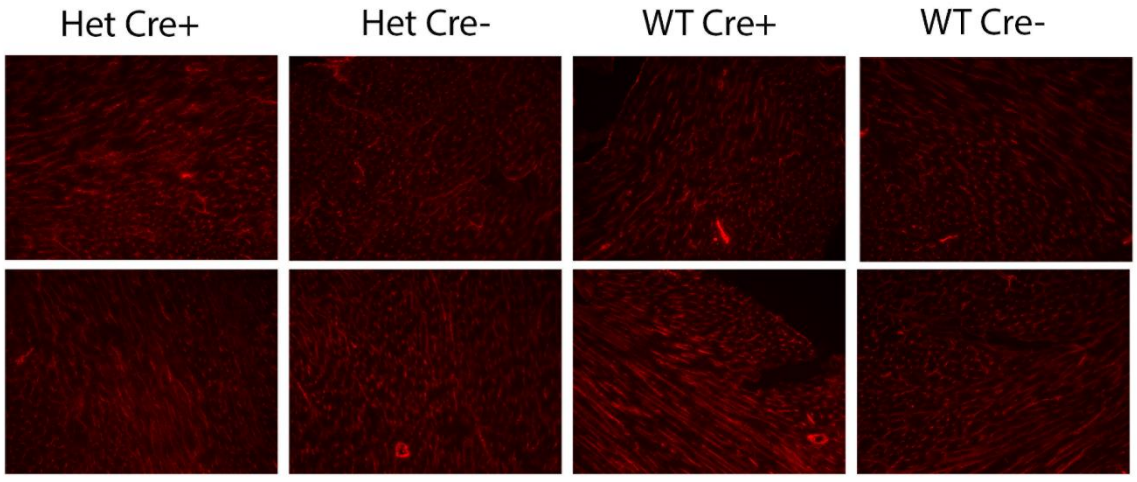


Figure 8: Transmission electron microscopy of CKD mouse hearts. The het cre+ mice showed a loss of electron density at the basal lamina. The basal lamina developed an abnormal wavy pattern and dissociated from the cardiomyocytes. This pattern was not seen in the WT cre+ mice, which shows that it was not caused by tamoxifen toxicity. Bar in the first three images = 2 µm and bar = 500 nm in last image

CD31 immunofluorescence is used to stain the endothelial cells in the vasculature in the heart. CD31 positive cells are shed from human cardiovascular disease into the bloodstream[14]. Since claudin-5 is present in both endothelial cells and cardiomyocytes claudin-5 may form a junction with CD31 positive cells. Therefore we examined CD31 immunofluorescence in the CKD heart samples. Our preliminary data may indicate that CD31 is slightly reduced in the het cre+ mice. Specifically, the microvasculature appears reduced and longitudinal regions show a waviness of the CD31 cells (see figure 9). This staining is similar to CD31 staining in dko mouse hearts, which exhibit cardiomyopathy (JRF lab, unpublished data). The abnormal membrane pattern matches the alterations in the basement membrane seen in the transmission electron microscopy.

Figure 9: Immunofluorescence staining of CD31 on CKD mouse hearts. CD31 is a marker of endothelial cells, particularly in the vasculature. Het cre+ mice show a reduction in microvasculature (the small circles). The het cre+ mice have also developed an abnormal wavy pattern in the longitudinal areas. In general, the CD31 staining is dimmer in the het cre+ mice, which indicates that CD31 positive cells may have been lost.



Discussion

The histological data shown here indicates that claudin-5 reduction results in major alterations to the basal lamina and extracellular matrix. These alterations are similar to those seen in the dko mouse model, which develops cardiomyopathy. Claudin-5 reduction may also result in CD31+ cell shedding; however this data is very preliminary and requires replication. These findings support my hypothesis that claudin-5 is necessary to maintain normal heart function and that claudin-5 reduction is a major step in the development of cardiomyopathy. While claudin-5 reduction alone has not yet caused reduced ejection fraction, a trend is beginning to appear and echocardiograms done at further time points may show reduced ejection fractions. This study shows the importance of examining the relationship between claudin-5 reduction and the development of cardiomyopathy. This study has shown that claudin-5 reduction is not simply correlated with cardiomyopathy, but that likely claudin-5 reduction is a step in the development of heart failure. While my lab showed that claudin-5 protein levels are reduced in end-stage human heart failure[15], recently claudin-5 gene expression was also shown to be reduced in human heart failure[12]. This mouse line can be used to further examine the relationship between claudin-5 and cardiomyopathy.

While the tamoxifen injected mice developed dilated hearts and had reduced whole heart function, this phenotype was likely due to a Cre-dependent toxicity, since both het cre+ and wt cre+ mice developed the phenotype. Tamoxifen citrate chow has been shown to be as effective as tamoxifen injection, while causing significantly less stress to the mice[11]. By switching our mice from tamoxifen injection to tamoxifen chow, we were able to alleviate the Cre-dependent toxicity. The tamoxifen chow mice develop a phenotype

more slowly than the tamoxifen injected mice. This is likely due to the fact that the tamoxifen chow mice are fed chow for two weeks, rather than one week of injection. Also, the slower development of a phenotype mimics the development of human cardiomyopathy more similarly. We will continue to perform longitudinal echocardiograms on the tamoxifen chow mice to determine if they develop reduced ejection fraction.

We still have to confirm why no floxed/floxed mice have been found in this mouse line. I will perform real-time quantitative PCR on these mice to test gene expression levels of claudin-5 and CDC45, a gene downstream of claudin-5 that may have been affected by the removal of the neo cassette. CDC45 is known to result in pre-implantational prenatal mortality[24]. Real-time PCR will also quantitatively show how much claudin-5 reduction we have achieved in this mouse model and allow us to compare this to the gene expression alterations in human heart failure.

I will also further analyze the cellular alterations quantitatively. Western blot analysis on subcellular fractions and isolated cardiomyocytes will allow me to examine claudin-5, ephrin-B1, and CD31 protein levels in both isolated membranes and cardiomyocytes. These experiments will provide quantitative support for the qualitative data shown here.

Lastly, additional studies of the effect of claudin-5 reduction on whole heart function can be performed. We will begin stressing the mice, using a β-adrenergic agonist, to determine if additional stress will exacerbate the phenotype. We can do a Kaplan-Meier survival analysis, to determine the effect of claudin-5 reduction on survival time, both with and without stress.

Both immunofluorescence and TEM indicate that claudin-5 reduction results in the development of abnormalities in the extracellular matrix. This indicates that claudin-5 could be involved with the mechanical attachment of the cardiomyocyte to the extracellular matrix. The reduction of claudin-5 would allow the cardiomyocytes to "slip" off of the matrix and then the matrix could begin to expand. This mechanism is supported by data shown in ephrin-B1 knockout mice. Ephrin-B1 is proposed to bind with claudin-5 and ephrin-B1 knockout mice have reduced claudin-5 and faulty matrix adhesion[6]. Another possible hypothesis is that claudin-5 binds to CD31+ cells, since claudin-5 is also present in endothelial cells. The loss of claudin-5 could destabilize the CD31+ cells and lead to the shedding of CD31+ cells into the bloodstream. After the CD31+ cells were lost, then the extracellular matrix would begin to proliferate and fibrosis could occur. To examine this mechanism, we plan to use flow cytometry to sort cells in the serum of this mouse line and to search for shed CD31+ cells.

Conclusion

The claudin-5 knockdown mouse model could potentially become a model for human dilated cardiomyopathy. These mice show many biochemical alterations similar to cardiomyopathy. This mouse line will be useful for future experiments examining the molecular mechanism behind the development of dilated cardiomyopathy. This mouse line also further supports the hypothesis that claudin-5 is necessary to maintain whole heart function. This mouse model can be used to develop various therapeutic targets and to further examine the therapeutic role of claudin-5.

References

1. Burke, AP "Dilated Cardiomyopathy Pathology" Medscape: Drugs, Diseases, & Procedures, 2013. World Wide Web. (http://emedicine.medscape.com/article/2017823- overview#aw2aab6b4) (March 13th, 2013)
2. Chun, JL, et al. "Cardiac Dysfunction and Cardiac dysfunction and pathology in the dystrophin and utrophin-deficient mouse during development of dilated cardiomyopathy" *Neuromuscul Disord,* 22(4):368-79, 2012.
3. Deconinck, AE, et al. "Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy." Cell, 90:717-727, 1997
4. Delfin, Dawn A., et al. "Sustaining cardiac claudin-5 levels prevents functional hallmarks of heart failure." Mol Ther, 20, 7:1378–1383, 2012
5. Dupont, E, et al. "Altered connexin expression in human congestive heart failure" J Mol Cell Cardiol. 33(2):359-371, 2001
6. Genet, G, Guilbeau-Frugier, C, et al. "Ephrin-B1 is a novel specific component of the lateral membrane of the cardiomyocyte and is essential for the stability of cardiac tissue architecture cohesion." Circ Res, 110:688-700, 2012
7. Hainsey, TA, et al. "Cardiomyopathic Features Associated with Muscular Dystrophy are Independent of Dystrophin Absence in Cardiovasculature." Neuromuscul Disord, 13:294-302, 2003.
8. Hewitt, KJ, Agarwal, R., and Morin, PJ. "The claudin gene family; Expression in normal and neoplastic tissues." BMC Cancer, 6:186, 2006

9. Janssen, PM., et al. “Utrophin Deficiency Worsens Cardiac Contractile Dysfunction Present in Dystrophin-Deficient Mdx Mice.” Am J Physiol Heart Circ Physiol, 289:H23732378, 2005.
10. Kaneshire, NK, et al. “Duchenne Muscular Dystrophy” Medline Plus, 2012. World Wide Web. (http://www.nlm.nih.gov/medlineplus/ency/article/000705.htm) (March 13th, 2013)
11. Kiermayer, C, et al. "Optimization of Spatiotemporal Gene Inactivation in Mouse Heart by Oral Application of Tamoxifen Citrate." Genesis, 45.1:11-16, 2007
12. Koczor, C.A, et al. “Detection of differentially methylated gene promoters in failing and nonfailing human left ventricle myocardium sing computation analysis” Physiol Genomics. 45: 597-605, 2013. Print
13. “Learning About Duchenne Muscular Dystrophy” National Human Genome Research Institute. World Wide Web. (http://www.genome.gov/19518854) (March 13th, 2013)
14. Mallat, Z., et al. “Elevated levels of shed membrane microparticles procoagulant potential in the peripheral circulating blood of patients with acute coronary syndrome” Circulation, 29; 101(8): 841-843, 2000
15. Mays, Tessily A., et al. "Claudin-5 levels are reduced in human end-stage Cardiomyopathy." J Mol Cell Cardiol, 45.1:81-7, 2008
16. McKenna, W.J., Perry, E. “Inherited Heart Conditions: Dilated Cardiomyopahy” British Heart Foundation, 2009. World Wide Web.

(http://www.cardiomyopathy.org/assets/files/BHF_DCM_FINAL1.pdf) (March 13th, 2013).

17. Morita, K, et al. "Endothelial claudin: claudin 5/TMVCF constitutes tight junction strands in endothelial cells" J Cell Bio, 147(1):185-94, 1999.
18. Mouse Mutant Resource Web Site. The Jackson Laboratory. Bar Harbor, Maine. World Wide Web (http://mousemutant.jax.org/) [Nov. 30, 2011]
19. Nitta, T., et al. "Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice." J Cell Biol, 161:653-660, 2003.
20. Quinlan, J.G., et al "Evolution of the mdx mouse cardiomyopathy: physiological and morphological findings" Neuromuscul Disord, 14(8-9):491-496, 2004.
21. Sanford, JL., et al. "Claudin-5 localizes to the lateral membranes of cardiomyocytes and altered in utrophin/dystrophin-deficient cardiomyopathic mice." J Mol Cell Cardiol, 38.2:323-332, 2005
22. Sohal, DS, et al. "Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein." Circ Res, 89:20-25, 2001
23. Wilmann, R., et al. "Mammalian animal models for Duchenne muscular dystrophy." Neuromuscul Disord, 4:241-249, 2009
24. Yoshida, K, et al. "Requirement of CDC45 for postimplantation mouse development" Mol Cell Biol, 21(14): 4598-603, 2001